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In vitro efficacy of ribavirin against canine distemper virus

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Abstract

Despite vaccination, canine distemper virus (CDV) remains one of the important pathogen of dogs with worldwide distribution. Ribavirin (RIB) inhibits replication of measles virus (MV), a morbillivirus closely related to CDV, both in vitro and in vivo. In this report the antiviral activity of RIB against CDV in cell cultures was assessed. Quantitative real-time RT-PCR was used to measure viral RNA in VERO cells infected by CDV and to evaluate the inhibitory effects of RIB. RIB caused a dose- and time-dependent decrease in accumulation of CDV RNA when added after virus adsorption. RIB was highly effective in preventing CDV replication at low concentrations with 50% virus-inhibitory concentrations ranging from 0.02 to 0.05 mM. Such low values were comparable to values displayed by highly susceptible strains of MV. In addition, CDV was passaged sequentially in VERO cell monolayers in the presence of RIB to trigger viral extinction. The virus was no longer detected after three passages, suggesting that error catastrophe is one of the modes of action of RIB against CDV. These findings suggest RIB as a promising tool for the therapy of CD in dogs.

Keywords: Antivirals; Ribavirin; Canine distemper virus; Real-time RT-PCR; VERO cells

1. Introduction

Canine distemper virus (CDV) is a single-stranded RNA virus in the genus Morbillivirus, family Paramyxoviridae, and is closely related to measles virus, rinderpest virus and phocine distemper virus. Biological and antigenic similarities among these viruses are well documented (Schobesberger et al., 2005). In dogs, CDV infection can result in subclinical infections, gastrointestinal signs and/or respiratory signs, frequently followed by central nervous system (CNS) involvement (Appel, 1987; Appel and Gillespie, 1972). Approximately 15% of canine inflammatory CNS diseases are caused by CDV (Appel and Summers, 1995). There are no specific antiviral agents for the treatment of CDV infection for, despite extensive vaccination, CDV infection remains a major disease of dogs.

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, RIB) is a purine nucleoside analog with a broad spectrum activity against both DNA and RNA viruses (Graci and Cameron, 2006). RIB has shown varying degrees of clinical efficacy against respiratory syncytial virus (Cooper et al., 2003), measles virus (Gururangan et al., 1990) and Lassa fever virus (Haas et al., 2003). In combination with pegylated interferon, RIB represents the standard therapy for the treatment of chronic hepatitis C infection (Fontanges et al., 2007).

The mechanisms by which RIB inhibits different viruses are difficult to elucidate since the drug appears to have pleiotropic effects. However, the antiviral mechanisms described for RIB can be traced back to the initial step of the drug's intracellular metabolism, e.g., the conversion of the RIB to ribavirin-5′-monophosphate (RMP) by adenosine kinase. This enzyme represents the intracellular rate-limiting step in RMP production and the variability of RIB antiviral activity in different cell lines has been attributed to phosphorylation differences (Parker, 2005).

RMP acts as a potent inhibitor of inosine monophosphate dehydrogenase (IMPDH) leading to the depression of intracellular guanosine triphosphate (GTP) levels (Streeter et al., 1973; Leyssen et al., 2005). The resulting nucleotide pool imbalance induces the viral polymerases to substitute alternative nucleosides for GTP, thus increasing the viral mutation frequency and production of defective genomes (Parker, 2005). RMP, moreover, undergoes subsequent phosphorylation and the final

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product, ribavirin-5′-triphosphate (RTP), represents the major intracellular active metabolite of RIB in mammalian cells.

Experimental evidence indicates that RTP can compete with ATP or GTP as an alternative substrate for the viral polymerase (Parker, 2005). Therefore, viral replication may be inhibited as a consequence of viral RNA chain termination. Since the inhibition of RNA elongation does not necessarily occur, anomalous viral genomes may be generated, accounting for the mutagenic effects described for RIB (Crotty et al., 2000; Contreras et al., 2002; Severson et al., 2003). Mutations may then accumulate as a virus goes through multiple rounds of replication, leading to inactivation of the viral population (error catastrophe).

The cellular metabolic products of RIB can reduce viral protein translation and RNA replication and also interfere with RNA capping efficiency (Bougie and Bisaillon, 2004; Hong and Cameron, 2002; Robins et al., 1985; Zhou et al., 2003). Furthermore, RIB has been shown to affect RNA replication by the direct inhibition of RNA polymerase (Wray et al., 1985), as observed with influenza virus, or by interfering with its activity (Eriksson et al., 1977).

In this study the inhibitory effects of RIB on CDV replication were assessed in vitro. A real-time RT-PCR assay (Elia et al., 2006) was used to evaluate the antiviral activity of RIB by quantification of viral RNA copies in CDV-infected VERO cells.

2. Experimental/materials and methods

2.1. Cells, virus and compounds

African green monkey kidney (VERO) cells (kindly provided by Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna-Brescia, Italy) were used throughout the experiments. Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum was used for cell growth and the same medium was used for antiviral assays. The Onderstepoort CDV vaccine strain was cultured in VERO cells to prepare a virus stock that was titrated by the endpoint dilution method and stored at −80 °C until used. For the experiments, RIB (Sigma–Aldrich Co., St Louis, MO, USA) was dissolved in distilled water, sterilized by filtration and diluted serially in two-fold increments in D-MEM in order to obtain compound concentrations from 0.03 to 16 mM.

2.2. Antiviral evaluation

Initial experiments were carried out to evaluate the inhibitory effect of RIB against CDV in VERO cells at 3 days post-infection (PI). Confluent monolayers of 24 h old VERO cells in 96-well plates were used. The medium was removed and the cells were infected with 40 tissue culture infectious doses ($TCID_{50}$) per well of the CDV stock. After 1 h of incubation at 37 °C, the inocula were removed and the monolayers were washed three times with D-MEM. The cell cultures were then incubated at 37 °C in the absence or presence of RIB at the stated concentrations. Four wells were used for each RIB dilution and all experiments were carried out in triplicate. At 24, 48 and 72 h PI, the supernatants and cryolysates from the wells corresponding to each RIB concentration, and from the untreated wells, were collected and the replicates were pooled. Each pool was then tested to evaluate the viral RNA load by using real-time RT-PCR for CDV.

2.3. Viral extinction

Viral extinction was monitored by serial passages of CDV in confluent monolayers of VERO cells. VERO cell monolayers were infected with CDV and RIB was added to the culture medium after virus adsorption, as described above. At 3 days PI, the untreated, infected cells exhibited clear cytopathic effect (CPE) and the supernatant fluids were collected. The supernatants of the four replicates were pooled and stored at −80 °C. Later, the virus was titrated by the end-point dilution method and 40 $TCID_{50}$ were used for subsequent passages. The supernatants of RIB-treated cells were also collected and the replicates were pooled. The same dilutions as with the untreated infected cells (1:1250) were used to re-infect the cells. The entire protocol was carried out on three separate 96-well plates and repeated for three consecutive passages. Samples (140 μl) of each pool were then tested by quantitative RT-PCR.

2.4. Quantitation of viral RNA load

Drug efficacy was evaluated by measuring viral RNA copies in samples collected from antiviral assays by a real-time quantitative RT-PCR. Total RNA was extracted from 140 μl cell culture supernatants and cryolysates using QIAamp Viral RNA methods (QIAamp Viral RNA, Qiagene S.pA., Italy), according to the manufacturer's instructions.

RNA was reverse transcribed in a reaction volume of 20 μl containing PCR buffer 1× (KCl 50 mM, Tris–HCl 10 mM, pH 8.3), $MgCl_2$ 5 mM, 1 mM of each deoxynucleotide (dATP, dCTP, dGTP, dTTP), RNase inhibitor 1 U, MuLV reverse transcriptase 2.5 U, random hexamers 2.5 U. Synthesis of c-DNA was carried out at 42 °C for 30 min, followed by a denaturation step at 99 °C for 5 min.

For construction of CDV standard RNA, a PCR assay targeting the nucleoprotein gene generated a 880 bp fragment that was cloned into pCR®4-TOPO (Invitrogen Srl, Milan, Italy) and in vitro transcribed (Elia et al., 2006). The transcript was then purified and quantified by spectrophotometrical analysis. Duplicates of the CDV standard dilutions and RNA templates were simultaneously subjected to real-time analysis, as previously described (Elia et al., 2006). Briefly, real-time PCR was carried out in a 50 μl reaction containing 25 μl of IQTM Supermix (Bio-Rad Laboratories Srl, Milan, Italy), 600 nM of primer CDV-F (5′-AGCTAGTTTCATCTTAACTATCAAATT-3′) and CDV-R (5′-TTAACTCTCCAGAAAACTCATGC-3′), 400 nM of probe CDV-Pb (FAMACCCAAGAGCCGGATACATAG-TTTCAATGC-TAMRA) and 20 μl of c-DNA.

Samples were analyzed on a 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) with parameters of 95 °C, 10 min (polymerase activation phase), followed by 45 cycles of denaturation at 95 °C for 15 s, primer annealing at 48 °C for 1 min and extension at 60 °C for 1 min.

2.5. Cytotoxicity assay

RIB was tested for cytotoxicity using a quantitative colorimetric neutral red (NR) assay (Borenfreund and Puerner, 1985). Briefly, confluent monolayers of VERO cells in 96-well plates were treated with serial two-fold dilutions of RIB in D-MEM (from 0.1 to 16 mM), using three wells per drug concentration, and then incubated at 37 °C, using the same experimental conditions as those used to evaluate drug antiviral activity. Each experiment was repeated three times. At 24, 48 and 72 h of incubation, the medium was removed and the cells were rinsed with PBS (pH 7.2). Two hundred and fifty microliters of NR (50 μg/ml) were then added to the monolayers and the cells were incubated for 3 h. Dye solution was removed and the monolayers were rinsed by a 2 min shaking with a wash-fix solution ($CaCl_2$ 1% in formaldehyde water solution 4%) and the retained stain was released by adding 100 μl/well of acetic acid/ethanol/water (1:50:49). After shaking for 20 min at room temperature, the supernatants were transferred to 96-well microtitre plates and read in an automatic spectrophotometer (microtitre plate reader, Bio-Rad 550) at a wavelength of 545 nm.

2.6. Data analysis

The amount of viral RNA in treated cultures was expressed as the percentage of the RNA in the non-treated CDV control cells. The relative cell viability observed in the cytotoxicity tests was expressed as the percentage of absorbance values of the samples versus the untreated control groups. After logarithmic conversion of drug and viral RNA copies, the data obtained in the antiviral activity assay were analysed by both a linear and non-linear curve fitting procedure (GraphPad Prism versus 3 software). The goodness of fit was tested by r^2 from non-linear regression and by the runs test. From the fitted dose–response curves obtained in each experiment, the potency was expressed as the 50% inhibitory concentration (IC_{50}) at which viral RNA copies decreased to half of that of cells cultured without addition of antiviral drug; the efficacy (E_{max}) is expressed as the maximum inhibitory effect of the drug on CDV replication in the different experimental conditions used.

A safety margin for VERO cells after 24 h of exposure to RIB was calculated as the ratio between the 20% cytotoxic concentration (CC_{20}) at which the viability of cells decreased to 80% of that of untreated cells and the 80% inhibitory concentration (IC_{80}). CC_{20} was calculated on the basis of linear regression of the percent reduction of cell viability at concentrations that ranged from 2.0 to 6.0 mM; the IC_{80} was calculated on the basis of non-linear regression of the concentration–antiviral efficacy curves. Results are expressed as the mean ± standard error of mean (S.E.M.) and the data were analyzed for statistical significance using the one-way ANOVA followed by Dunnet's multiple comparison test. Differences were taken as significant when $P < 0.05$.

3. Results

3.1. Antiviral evaluation

VERO cells infected with CDV and incubated for 72 h in media containing various concentrations of RIB showed that RIB inhibited CDV replication in a dose-dependent fashion (Fig. 1). At the highest concentration (1.0 mM), inhibition of the intracellular virus was greater than 2.0, 3.0 and 5.0 log $TCID_{50}$ at 24, 48 and 72 h after exposure, respectively. Log RNA copies/ml of the untreated control cultures, plotted against the incubation time, revealed a direct relationship between viral RNA yields and the incubation time (linear regression: $r^2 = 0.999$). The relationship between drug effects and the times of exposure showed that antiviral activity at the higher concentrations (1.0–0.5 mM) was not related to the time interval of exposure since maximal efficacy was observed after the 24 h period. However, at lower concentrations (starting from 0.06 mM = 15 μg/ml) the drug efficacy was dose-related and using 0.25–0.12 mM concentrations, the maximal effect was obtained only after a 48 h exposure (Fig. 2).

Analysis of the supernatant fluid samples collected from the control cells revealed that virus titres in the supernatants were about 2–5% the virus titres in the intracellular compartment (1.8, 4.5 and 5.2% at 24, 48 and 72 h of incubation, respectively). Starting from 48 h exposure to RIB, there was a dose-dependent ($r^2 > 0.9$) decrease in the supernatant virus load. After 24 h of exposure no drug dose–efficacy relationship was recorded ($r^2 = 0.45$) and viral titres did not exceed more than 50% of the virus amounts in the infected control cells (Fig. 1).

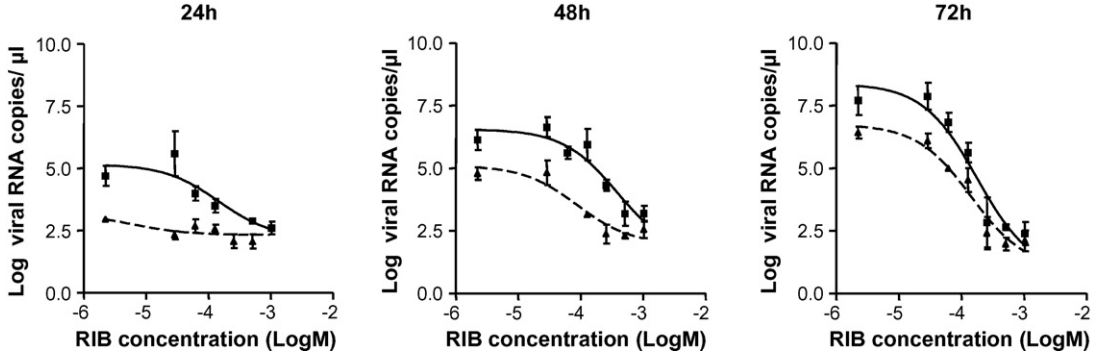

Fig. 1. Non-linear regression of the concentration–effect curves showing the antiviral activity of ribavirin against canine distemper virus replication as assessed by real-time RT-PCR on cryolysate (continuous line) and supernatant (dotted line) at different exposure times. Data are expressed as mean ± standard error of mean (S.E.M.).

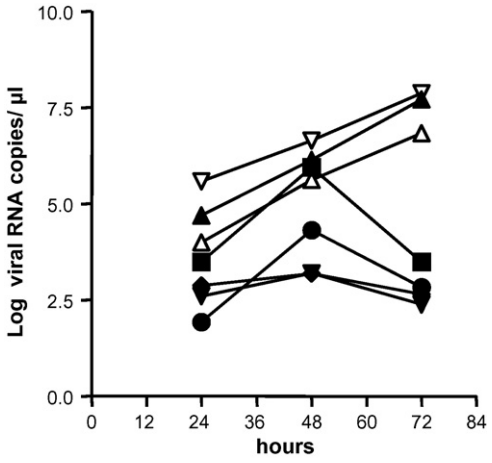

Fig. 2. Antiviral effect of ribavirin against canine distemper virus replication in VERO cells as assessed by real-time RT-PCR at three different incubation times. (▽) control; (▲) 0.03 mM; (△) 0.06 mM; (■) 0.12 mM; (●) 0.25 mM; (▼) 0.5 mM; (♦) 1.0 mM. Data are expressed as mean without standard error of mean (S.E.M.) that, however, never exceeded 15% of the mean values.

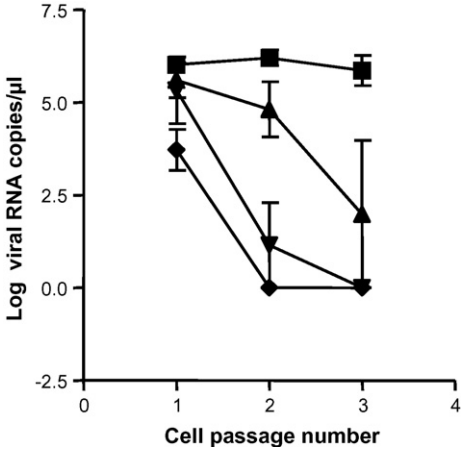

Fig. 3. Virus titre after sequential passages in VERO cells exposed to ribavirin at different concentrations (■, control; ▲, 0.03 mM; ▼, 0.06 mM; ♦, 0.12 mM). Data are expressed as mean ± standard error of mean (S.E.M.).

Table 1 shows the potency (IC_{50}) and efficacy (E_{max}) parameters of RIB activity, together with the IC_{80} values, calculated by the fitted dose–response curves and the goodness of drug dose–efficacy relationship (r^2).

3.2. Viral extinction

Viral extinction was evaluated by sequential passages of CDV in RIB-treated cells. At the lowest concentration (0.03 mM) viral RNA copies decreased proportionally over the various passages; by the third passage the mean RNA copy number was significantly (about five-fold) lower ($P < 0.05$) than in the untreated infected cells. Complete viral extinction was observed after three passages in the cells treated with 0.06 mM RIB and at the second passage in the cells treated with ≥0.12 mM RIB (Fig. 3). Viral RNA copies in the untreated cells remained constant over the three passages.

3.3. Cytotoxicity assay

No significant toxicity was observed in VERO cells exposed to the drug at concentrations that varied between 0.4 and 4.0 mM. However, significant cytotoxic effects ($P < 0.01$) were observed at 24 h post-exposure at concentrations ≥6.0 mM. Control cell viability decreased by nearly 30% with every two-fold increase in RIB concentration. Significant cytotoxicity ($P < 0.05$) was observed only at the highest concentrations (16 mM), with 20 and 15% decreases in cellular viability after 48 and 72 h exposure, respectively.

RIB safety margin of 57.66 for VERO cells after 24 h of exposure was calculated as the CC_{20}/IC_{80} ratio (5.19/0.09 mM).

4. Discussion

Although known for centuries, CDV still remains a major pathogen of dogs with worldwide distribution. The canine virus is closely related to measles virus (MV) (Adams and Imagawa, 1957) and this relationship may be exploited to compare or evaluate in parallel the efficacy of molecules of potential therapeutic interest. Since RIB has been reported to have superior antiviral effects against MV than other nucleoside and non-nucleoside compounds (Hosoya et al., 1989), it was of interest to assess whether RIB could also inhibit CDV replication in vitro.

In order to evaluate the effects of antiviral drugs in cell cultures, it is necessary to determine their ability to inhibit virus replication at non-cytotoxic concentrations and to ascertain their concentration-dependent activities. Titration of infectious virus in cell cultures is usually achieved by the end-point dilution method which is time consuming. Evaluation of viral growth may be based on the observation of CPE, but it is sometimes difficult to distinguish virus-induced CPE from non-specific cell alterations. In this study, we evaluated RIB inhibitory effects

Table 1
Antiviral potency (IC_{50}–IC_{80}) and efficacy (E_{max}) of ribavirin against CDV in VERO cells

Exposure time (h)	Cryolysates				Supernatants			
	r^2	IC_{50}[a] (mM)	IC_{80} (mM)	E_{max}[b] (%)	r^2	IC_{50}[a] (mM)	IC_{80} (mM)	E_{max}[b] (%)
24	0.9	0.05 ± 0.007	0.09 ± 0.01	99.89	0.45	–	–	–
48	0.9	0.06 ± 0.005	0.11 ± 0.01	100	0.91	0.022 ± 0.001	0.042 ± 0.002	100
72	0.93	0.02 ± 0.001	0.04 ± 0.002	100	0.95	0.017 ± 0.001	0.031 ± 0.002	100

[a] IC_{50}–IC_{80} values are expressed as mean ± S.E.M. The IC_{50} and IC_{80} are defined as the concentration at which viral RNA copies decreased to 50 and 80% of that of cells cultured without addition of antiviral drug.

[b] E_{max} is expressed as the percentage decrease of viral RNA copies vs. infected non-treated cells.

on CDV by quantitation of viral RNA copies in real-time RT-PCR assays. Compared to conventional methods, the real-time RT-PCR was faster and more objective.

Evidence has been provided that RIB exerts its activity against MV with IC_{50} between 8.6 and 66.7 μg/ml, with the differences depending on the virus strain and/or experimental conditions (Shigeta et al., 1992; Wyde et al., 2000; Grancher et al., 2004). In our experiments, RIB proved to be highly effective in preventing CDV replication at low concentrations, ones that ranged from 0.02 to 0.05 mM IC_{50} (6.5–12.5 μg/ml). Such values are similar to those obtained in tests of RIB against highly susceptible MV strains.

As suggested in the cytotoxicity tests, actual RIB antiviral effects likely accounted for decreases in the viral titres we observed in the antiviral assays since RIB-induced cellular toxicity was not observed within the effective concentration range of RIB.

The in vitro antiviral activity of RIB varies depending on the cell line used (Sidwell, 1980), a phenomenon that is likely due to phosphorylation differences (Smee et al., 2001). Unlike previous reports (Smee et al., 2001; Cinatl et al., 2005), the efficacy of RIB in VERO cells suggests sufficient phosphorylation of the drug to its active forms in simian cells.

RIB also was shown to inhibit CDV replication in vitro in a dose- and time-dependent manner. Even though non-cytotoxic drug concentrations of 0.5–1 mM completely blocked CDV replication 24 h after RIB exposure, the timing experiments indicate that the IC_{50} value was twice as high at 24 h (0.05 mM) as at 72 h post exposure (0.02 mM). This would suggest that RIB affects virus transit into the extracellular compartment early in the viral replication cycle. After 24 h exposure, in fact, even at low RIB concentrations, in the treated cells a low number of viral RNA copies was observed, suggesting a decrease in the assembly of new virus particles able, subsequently, to migrate into the extracellular compartment and to spread to other cells.

The viral extinction studies indicate that RIB at the lowest concentrations (0.03–0.12 mM) significantly affects CDV replication. This finding suggests an accumulation of detrimental mutations in the viral genome (error catastrophe).

Since RIB has various mechanisms of antiviral action, it is difficult to identify a specific antiviral activity (Parker, 2005; Day et al., 2005; Leyssen et al., 2005). The experimental design adopted in the present study could not fully examine the modes of antiviral action exploited by RIB. However, our findings showed, at low drug concentrations, that the viral RNA copies detected by real-time RT-PCR correlate with low virus infectivity and that the antiviral activity of RIB is time-dependent.

Since, as observed with influenzavirus, the concentration of RTP required for incorporation into the viral genome is much less than that required to inhibit RNA elongation and since the viral RNA copies containing fraudulent nucleotides by means of inhibition of IMPDH early appear (Parker, 2005), it may be speculated that RIB interferes with CDV RNA polymerase by competing with natural nucleosides and producing the error catastrophe phenomena rather than by causing chain termination.

Since RIB efficacy in the infected cells increased with the exposure time, it can be accounted for by the asynchronous activation of various mechanisms (e.g., increase in mutation frequency, viral RNA termination, interference with RNA capping, etc.).

By the NR assay, relatively low cytotoxicity was displayed by RIB in confluent VERO cells since drug-induced CPE was detectable only at concentrations $\geq$6.0 mM. Moreover, using the concentrations adopted for the cytotoxicity study, a $CC_{50} > 16$ mM was estimated. Our results do not allow definition of the RIB selectivity index (CC_{50}/IC_{50}). However, the safety margin, calculated as the ratio CC_{20}/IC_{80} at the 24 h exposure time, produced the highest drug toxicity, indicating that RIB induces 20% cell mortality at concentrations 50 times higher than the concentrations required to cause 80% decrease in the viral RNA copies. Several compounds have been shown to have little or no toxicity in confluent cell monolayers, but there was considerable toxicity in freshly seeded cells (Baker et al., 2003). According to our findings and to previous experiments (Shigeta et al., 1992; Kimura et al., 2000; Grancher et al., 2004), RIB toxicity in VERO cells is likely to be affected by the cell replicative activity.

RIB-elicited toxicity in VERO cells was inversely correlated with the times of exposure since exposure to this compound for short periods (e.g., 24 h) resulted in more severe cytotoxic effects. These findings give additional support to our hypothesis on the mechanism of RIB action against CDV since RIB cytotoxicity is due to the inhibition of IMPDH by RMP and RTP is not a substrate for cellular polymerase (Parker, 2005).

In conclusion, evidence presented in this study indicates that RIB has strong inhibitory effects on CDV replication in vitro, encouraging hope for its clinical applicability in vivo. The time-dependant antiviral activity and the effectiveness at low concentrations may justify in vivo studies aimed at determining therapeutic tissue levels, i.e., ones lower than the estimated IC_{50} which could result in better drug tolerance and be used in dogs over prolonged periods.

References

Adams, J.M., Imagawa, D.T., 1957. Immunological relationship between measles and distemper viruses. Proc. Soc. Exp. Biol. Med. 96, 240–244.

Appel, M.J., 1987. In: Appel, M.J. (Ed.), Virus Infection of Carnivores. Elsevier, Amsterdam, pp. 133–159.

Appel, M.J., Gillespie, J.H., 1972. Canine distemper virus. Virol. Monogr. 11, 1–96.

Appel, M.J., Summers, B.A., 1995. Pathogenicity of morbilliviruses for terrestrial carnivores. Vet. Microbiol. 44, 187–191.

Baker, R.O., Bray, M., Huggins, J.W., 2003. Potential antiviral therapeutics for smallpox, monkeypox and other orthopoxvirus infections. Antiviral Res. 57, 13–23.

Borenfreund, E., Puerner, J.A., 1985. Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicol. Lett. 24, 119–124.

Bougie, I., Bisaillon, M., 2004. The broad spectrum antiviral nucleoside ribavirin as a substrate for a viral RNA capping enzyme. J. Biol. Chem. 279, 22124–22130.

Cinatl, J., Michaelis, M., Hoever, G., Preiser, W., Doerr, H.W., 2005. Development of antiviral therapy for severe acute respiratory syndrome. Antiviral Res. 66, 81–97.

Contreras, A.M., Hiasa, Y., He, W., Terella, A., Schmidt, E.V., Chung, R.T., 2002. Viral RNA mutations are region specific and increased by ribavirin in a full-length hepatitis C virus replication system. J. Virol. 76, 8505–8517.

Cooper, A.C., Banasiak, N.C., Allen, P.J., 2003. Management and prevention strategies for respiratory syncytial virus (RSV) bronchiolitis in infants and young children: a review of evidence-based practice interventions. Pediatr. Nurs. 29, 452–456.

Crotty, S., Maag, D., Arnold, J.J., Zhong, W., Lau, J.Y., Hong, Z., Andino, R., Cameron, C.E., 2000. The broad-spectrum antiviral ribonucleoside ribavirin is an RNA virus mutagen. Nat. Med. 6, 1375–1379.

Day, C.W., Smee, D.F., Julander, J.G., Yamshchikov, V.F., Sdiwell, R.W., Morrey, J.D., 2005. Error-prone replication of West Nile virus caused by ribavirin. Antiviral Res. 67, 38–45.

Elia, G., Decaro, N., Martella, V., Cirone, F., Lucente, M.S., Lorusso, E., Di Trani, L., Buonavoglia, C., 2006. Detection of canine distemper virus in dogs by real-time RT-PCR. J. Virol. Methods 136, 171–176.

Eriksson, B., Helgstrand, E., Johansson, N.G., Larsson, A., Misiorny, A., Noren, J.O., Philipson, L., Stenberg, K., Stening, G., Stridh, S., Oberg, B., 1977. Inhibition of influenza virus ribonucleic acid polymerase by ribavirin triphosphate. Antimicrob. Agents Chemother. 11, 946–951.

Fontanges, T., Beorchia, S., Douvin, C., Delassalle, P., Combis, J.M., Hanslik, B., Jacques, J.P., Filoche, B., Desmorat, H., Chandelier, C., Ouzan, D., 2007. Safety and efficacy of combination therapy with peginterferon alfa-2a (40 kDa) and ribavirin in the outpatient setting: prospective analysis of 197 patients with chronic hepatitis C viral infection. Gastroenterol. Clin. Biol. 31, 566–572.

Graci, J.D., Cameron, C.E., 2006. Mechanisms of action of ribavirin against distinct viruses. Rev. Med. Virol. 16, 37–48.

Grancher, N., Venare, V., Kedzierewicz, F., Ammerlaan, W., Finance, C., Muller, C.P., Le Faou, A., 2004. Improved antiviral activity in vitro of ribavirin against measle virus after complexation with cyclodextrins. Antiviral Res. 62, 135–137.

Gururangan, S., Stevens, R.F., Morris, D.J., 1990. Ribavirin response in measles pneumonia. Infection 20, 219–221.

Haas, W.H., Breuer, T., Pfaff, G., Schmitz, H., Köhler, P., Asper, M., Emmerich, P., Drosten, C., Gölnitz, U., Fleischer, K., Günther, S., 2003. Imported Lassa fever in Germany: surveillance and management of contact persons. Clin. Infect. Dis. 36, 1254–1258.

Hong, Z., Cameron, C.E., 2002. Pleiotropic mechanisms of ribavirin antiviral activities. Prog. Drug Res. 59, 41–69.

Hosoya, M., Shigeta, S., Nakamura, K., De Clercq, E., 1989. Inhibitory effect of selected antiviral compounds on measles (SSPE) virus replication in vitro. Antiviral Res. 12, 87–97.

Kimura, K., Mori, S., Tomita, K., Ohno, K., Takahashi, K., Shigeta, S., Terada, M., 2000. Antiviral activity of NMSO3 against respiratory syncytial virus infection in vitro and in vivo. Antiviral Res. 47, 41–141.

Leyssen, P., Balzarini, J., De Clercq, E., Neyts, J., 2005. The predominant mechanism by which ribavirin exerts its antiviral activity in vitro against Flaviviruses and Paramyxoviruses is mediated by inhibition of IMP dehydrogenase. J. Virol. 79, 1943–1947.

Parker, W.B., 2005. Metabolism and antiviral activity of ribavirin. Virus Res. 107, 165–171.

Robins, R.K., Revankar, G.R., McKernan, P.A., Murray, B.K., Kirsi, J.J., North, J.A., 1985. The importance of IMP dehydrogenase inhibition in the broad spectrum antiviral activity of ribavirin and selenazofurin. Adv. Enzyme Regul. 24, 29–43.

Schobesberger, M., Summerfield, A., Doherr, M.G., Zurbriggen, A., Griot, C., 2005. Canine distemper virus-induced depletion of uninfected lymphocytes is associated with apoptosis. Vet. Immunol. Immunopathol. 104, 33–44.

Severson, W.E., Schmaljohn, C.S., Javadian, A., Jonsson, C.B., 2003. Ribavirin causes error catastrophe during Hantaan virus replication. J. Virol. 77, 481–488.

Shigeta, S., Mhori, S., Baba, M., Ito, M., Honzumi, K., Nakamura, K., Oshitani, H., Numazaki, Y., Matsuda, A., Obara, T., Shuto, S., de Clercq, E., 1992. Antiviral activity of ribavirin, 5-ethynyl-1-β-D-ribofuranosilimidazole-4-carboxamide, and 6′-(*R*)-6′-C-methylneplanocin A against several *ortho*- and paramyxoviruses. Antimicrob. Agents Chemother. 36, 435–439.

Sidwell, R.W., 1980. Ribavirin: in vitro antiviral activity. In: Smith, R.A., Kirkpatrick, W (Eds.), Ribavirin: A Broad Spectrum Antiviral Agent. Academic Press Inc., New York, pp. 23–42.

Smee, D.F., Bray, M., Huggins, J.W., 2001. Antiviral activity and mode of action studies of ribavirin and mycophenolic acid against orthopoxviruses in vitro. Antiviral Chem. Chemother. 12, 327–335.

Streeter, D.G., Witkowski, J.T., Gyaneshwar, P.K., Sidwell, R.W., Bauer, R.J., Robins, R.K., Simon, L.N., 1973. Mechanism of action of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole), a new broad-spectrum antiviral agent. Proc. Natl. Acad. Sci. U.S.A. 70, 1174–1178.

Wray, S.K., Gilbert, B.E., Knight, V., 1985. Effect of ribavirin triphosphate on primer generation and elongation during influenza virus transcription in vitro. Antiviral Res. 5, 39–48.

Wyde, P., Moore-Poveda, D.K., De Clercq, E., Neyts, J., Matsuda, A., Minakawa, N., Guzman, E., Gilbert, B.E., 2000. Use of cotton rats to evaluate the efficacy of antivirals in treatment of measle virus infections. Antimicrob. Agents Chemother. 44, 1146–1152.

Zhou, S., Liu, R., Baroudy, B.M., Malcolm, B.A., Reyes, G.R., 2003. The effect of ribavirin and IMPDH inhibitors on hepatitis C virus subgenomic replicon RNA. Virology 310, 333–342.